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14. ABSTRACT
Inflammatory breast cancer (IBC) is a highly aggressive form of locally advanced breast cancer that is often characterized by ErbB2 and ErbB1 overexpression. ErbB-targeting is clinically relevant using trastuzumab, an anti-ErbB2 antibody, and lapatinib, a small molecule ErbB1/2 kinase inhibitor. However, acquired resistance is common even in those patients who show an initial clinical response; this resistance is in part due to apoptotic dysregulation, which allows transformed cells to survive and proliferate, even in the presence of therapeutics. In part, this failure is due to defects in caspase activity, the execution phase of apoptosis. X-linked inhibitor of apoptosis protein (XIAP) is a potent anti-apoptotic protein that is capable of inhibiting both the mitochondrial and extrinsic apoptotic pathways by binding caspases, which inhibits their activation. In studies conducted to date, we have generated both ErbB2 overexpressing SUM190 and ErbB1 activated SUM149 IBC cells with stable XIAP overexpression and shown that this can reverse sensitivity of these cells to GW583340, a lapatinib analog. Additionally, we have generated GW583340 resistant IBC lines (rSUM190 and rSUM149) and characterized expression of pro-survival and anti-apoptotic proteins in these cells. We have identified overexpression of XIAP in acquired resistance to GW583340 in both SUM190 and SUM149 IBC cell lines.

15. SUBJECT TERMS
Inflammatory breast cancer, XIAP, apoptosis, ErbB2 targeting agents

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Annual Report (July 1, 2008-June 30, 2009)

Introduction

Inflammatory breast cancer (IBC) is a highly aggressive form of locally advanced breast cancer that is often characterized by ErbB2 and ErbB1 overexpression. ErbB-targeting is clinically relevant using trastuzumab, an anti-ErbB2 antibody and lapatinib, a small molecule ErbB1/2 kinase inhibitor. However, acquired resistance is a common outcome even in those IBC patients who show an initial clinical response; this resistance is in part due to apoptotic dysregulation. Apoptotic dysregulation is a fundamental characteristic of cancer that allows transformed cells to survive and proliferate, even in the presence of therapeutic agents. In part, this failure is due to defects in caspase activity, which is the execution phase of apoptosis. X-linked inhibitor of apoptosis protein (XIAP) is one of the most potent anti-apoptotic proteins and is capable of inhibiting both the mitochondrial and extrinsic pathways of apoptosis by binding to caspases, which inhibits their cleavage and subsequent activation. The aims of this proposal are, 1) To evaluate the mechanism of XIAP in inhibiting apoptosis in acquired resistance to ErbB1 and ErbB2 targeting strategies in ErbB2 overexpressing (SUM190) and ErbB1 activated (SUM149) IBC cell lines; 2) Development and characterization of novel XIAP inhibitors alone and in combination with trastuzumab and GW583340 (a lapatinib analog) in an in vivo SUM190 xenograft imageable tumor model. In studies conducted to date, we have generated both SUM190 and SUM149 cells with stable XIAP overexpression and shown that this can reverse sensitivity of these cells to GW583340. Additionally, we have generated two GW583340 resistant IBC lines (rSUM190 and rSUM149) and characterized expression of pro-survival and anti-apoptotic proteins in these cells. We have identified overexpression of the anti-apoptotic protein XIAP in acquired resistance to GW583340 in both ErbB2 overexpressing SUM190 and ErbB1 activated SUM149 cell lines derived from primary IBC tumors.

Body

(The progress report is presented as results of tasks outlined for year 1 in the original Statement of Work.)

Task 1. Determine the mechanism of XIAP action in conferring resistance to Trastuzumab and GW583340. For this two IBC cell lines will be used- an ErbB2 overexpressing IBC cell line (SUM190) and an ErbB1 activated IBC cell line (SUM149).

A. XIAP overexpression studies in SUM190 and SUM149 cells-compare and contrast the parental and XIAP overexpressed cells for resistance to Trastuzumab and GW583340.

A previous study in our lab (1) has observed the following:

1. Sensitivity of IBC cells to trastuzumab and GW583340 correlates with decrease in XIAP expression.
2. Overexpression of endogenous XIAP correlates with resistance of SUM190 IBC cells to trastuzumab.

Hypothesis/Objectives: XIAP is a novel molecular sensor that plays a critical role in the failure of IBC cells to undergo apoptosis and in conferring a therapeutic resistant phenotype in IBC. As resistance to ErbB1/ErbB2 inhibitors seems to correlate with increased XIAP expression in IBC, the objectives of Task 1 are to:

1. Determine whether overexpression of XIAP in ErbB2 overexpressing IBC cells and triple negative/ErbB1 activated IBC cells can reverse sensitivity to GW583340.
2. Understand the mechanism of apoptotic dysregulation in these IBC cells in response to Trastuzumab and Lapatinib.

Table 1. The IBC cellular model used in this study.

Cell Line	ErbB2 expression	ErbB1 expression	Trastuzumab	GW583340
SUM190	Overexpressed, high p-ErbB2	Low	Resistant (1)	Sensitive (1-3)
SUM149	Low	Activated	Resistant (1)	Sensitive (3, 4)
rSUM190	Overexpression, no p-ErbB2	Low	Resistant	Resistant (2.5 μM) (3)
rSUM149	Low	Low p-ErbB1	Resistant	Resistant (7.5 μM) (3)

Methods and Results

XIAP overexpression plasmids

In addition to the Flag-tagged XIAP plasmid the PI lab had in hand, we have also received XIAP

lentiviral expression plasmids from Dr. Colin Duckett's lab (University of Michigan), which can be used to stably overexpress wildtype and mutant forms of XIAP (Fig. 1) (5). Moreover, these plasmids decrease transfection reagent-related cytotoxicity from transient transfections and will allow for a more thorough investigation of the mechanism of XIAP biology in trastuzumab and GW583340 resistance.

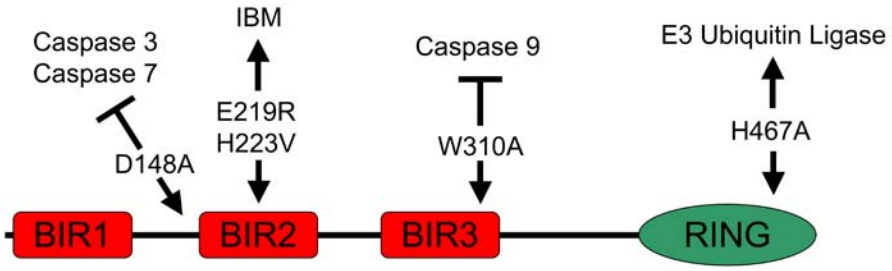

Figure 1. Schematic of various point mutants of XIAP. D148A disrupts interaction with caspases 3 and 7. E219R H223V disrupts interaction with inhibitory proteins that bind XIAP with an IBM (IAP binding motif). W310A prevents interaction with caspase 9. H467A disrupts the E3 ubiquitin ligase activity of XIAP. The double mutant D148A/W310A cannot bind to caspase 3, 7, or 9.

<u>Optimizing overexpression of XIAP in IBC cells</u>

To date, both SUM149 and SUM190 IBC cells have been stably transfected with shXIAP and wtXIAP (along with their respective vector controls) (Fig. 2).

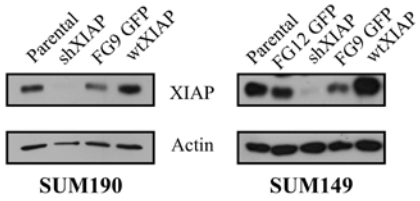

Figure 2. Expression of XIAP in stably transfected SUM190 and SUM149 IBC cells.

Methods to generate these stable cells: FG12 GFP, shXIAP, FG9 GFP, and wtXIAP were cotransfected with pHCMV-G, pRRE, and pRSVrev (6), which direct the expression of lentiviral structural proteins, into HEK293T cells using Lipofectamine 2000 and incubated at 37ºC, 5 % CO_2. Forty hours post-transfection, the virus-containing media on the HEK293T cells was collected, polybrene was added (25 mM), and the media was filtered through a .45 mm filter unit onto SUM149 cells. Stable cell lines were selected by FACS sorting for GFP expression (FG12 GFP and shXIAP; Fig. 3) or hygromycin selection (FG9 GFP and wtXIAP).

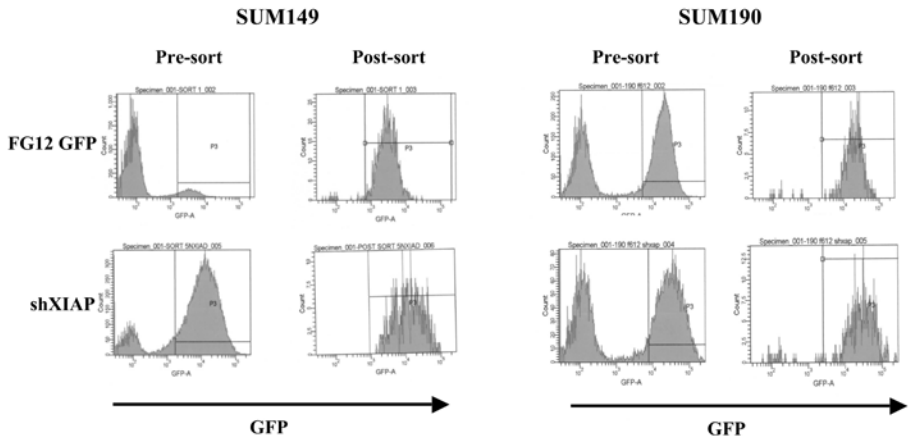

Figure 3. Flow cytometric data showing GFP expression in SUM149 and SUM190 IBC cells stably expressing GFP or shXIAP before sorting (left) and after sorting (right).

XIAP overexpression protects IBC cells from GW583340-induced cytotoxicity

Our previous data indicated that sensitivity to GW583340 directly correlated with XIAP expression and therefore we wanted to determine whether overexpression of XIAP could reverse sensitivity to GW583340. SUM190 and SUM149 parental, vector control (FG9 GFP), and wtXIAP overexpressing cells were treated with GW583340 for 24 h and cell death was assessed by staining with Aqua Vivid (AqVID, Invitrogen), which can only get into cells whose membrane integrity is lost. Results in Figure 4 show that overexpression of wtXIAP in both SUM190 and SUM149 cells significantly reversed GW583340-mediated cytotoxicity.

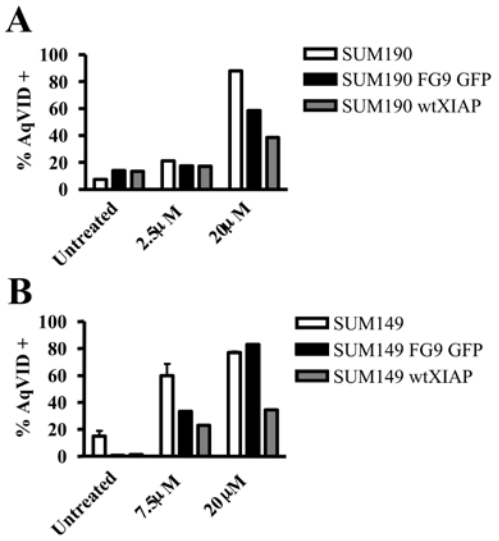

Figure 4. Percentage of dead cells (AqVID +) in parental and stably transfected (vector control and wtXIAP) IBC cells treated with lapatinib for 24 h.

B. Expression analysis of key anti-apoptotic and pro-survival factors to determine critical factor in resistance of SUM190 and SUM149 cells to GW583340 (a dual ErbB1/2 tyrosine kinase inhibitor).

3. IBC tumors often display overexpression of ErbB1 (7) or ErbB2 (8) and therefore lapatinib (a dual ErB1/2 tyrosine kinase small molecule inhibitor; (9)) is often used in this subset of patients.
4. Although IBC patients initially respond well to lapatinib (10, 11), sensitivity is short-lived and acquired resistance is common (12, 13).

Objectives: The objectives of Task 2 are to:

1. Generate a GW583340-resistant cellular model.

2. Determine expression analysis of anti-apoptotic and pro-survival proteins.

Methods and Results

Generation of GW583340-analog-resistant IBC cell lines:

To make lapatinib-analog (GW583340)-resistant cell lines, parental SUM190 and SUM149 IBC cells (Table 1) were cultured in increasing concentrations of GW583340 [0.25 μM- 2.5 μM (SUM190) or 0.25 μM-7.5 μM (SUM149)] for more than three months. At first, massive cell death was observed, but after repeated culture in the drug, a resistant clonal population grew. These resistant cells (rSUM190 and rSUM149) had similar doubling times to their parental counterparts and baseline apoptosis was the same as untreated parental cells (Fig. 5).

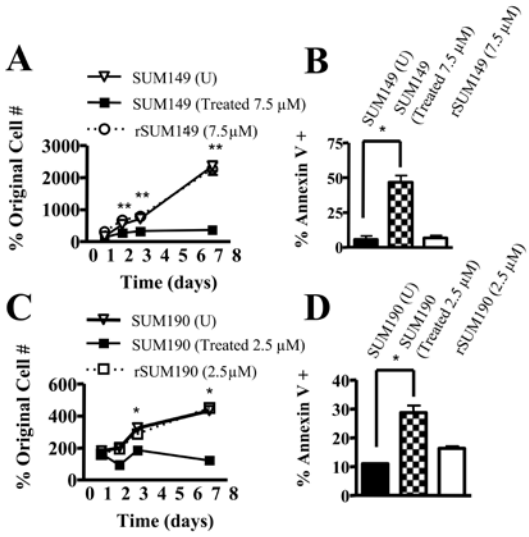

Figure 5. Lapatinib-resistant cells have the same doubling time as their parental untreated counterparts and less cell death and apoptosis than the parental cells treated at the same concentration.

Characterization of ErbB1/2 signaling pathway in parental and resistant IBC cells

Evaluation of the effect of GW583340 on the ErbB signaling pathways in SUM190 and SUM149 IBC cells revealed that treatment with GW583340 caused a marked and comparable downregulation of p-ErbB1 in both parental SUM149 and resistant rSUM149 cells compared to untreated parental cells (Fig. 6). Similarly, expression of p-ErbB2, p-AKT, and p-MAPK were inhibited in the GW583340-treated SUM190 and resistant rSUM190 cells, along with an increase in total MAPK expression (Fig. 6). These data suggest that the primary mechanism of GW583340 action as a dual ErbB1/2 kinase inhibitor (14) is not compromised in the resistant rSUM190 and rSUM149 cells.

Characterization of anti-apoptotic proteins in parental and resistant IBC cells

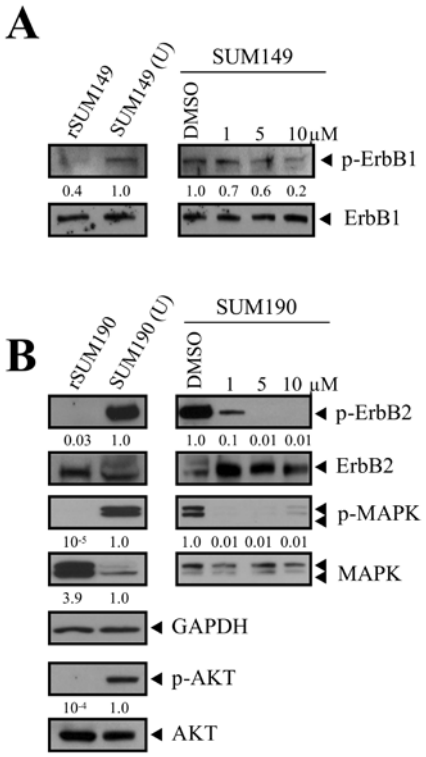

Figure 6. Western blot analysis of ErbB1 and ErbB2 signaling pathways in parental and resistant IBC cells.

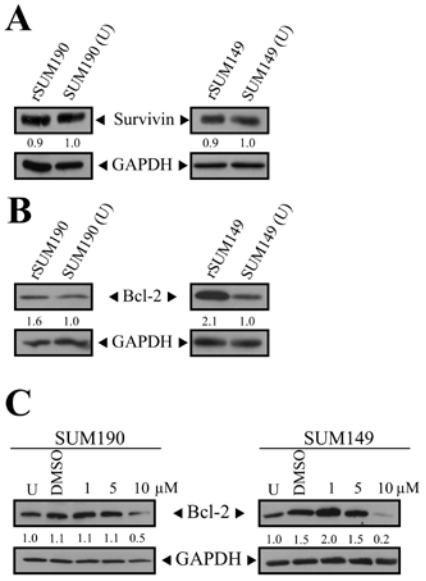

Figure 7. Western blot analysis of anti-apoptotic proteins survivin and Bcl-2.

A previous report (15, 16) in non-IBC cells and patients demonstrated that sensitivity to lapatinib correlates with inhibition of survivin, a member of the IAP family. Additionally, our lab has shown that increased XIAP correlates with resistance to trastuzumab (another ErbB2 targeted agent) in SUM190 IBC cells (1). Therefore, immunoblot analyses of GW583340 treated lysates for key anti-apoptotic proteins (survivin, XIAP, procaspase 9 and Bcl-2) were conducted. Data in Figure 7 reveal that survivin expression was not inhibited in the resistant IBC cells, consistent with previous studies in a non-IBC breast cell line (BT474) (15) and an ovarian carcinoma cell line (PEO1) (17), wherein high survivin expression was sustained in cells resistant to GW583340.

Data in Figure 7 also show that rSUM190 and rSUM149 cells had high Bcl-2 protein levels in comparison to the untreated parental cells. In contrast, a significant decrease in Bcl-2 expression was observed post-GW583340 treatment in the parental SUM190 and SUM149 cells undergoing apoptosis (Fig. 5).

Immunoblot analysis of XIAP protein levels (Fig. 8) in the IBC cells showed a 2-3 fold overexpression of XIAP in both rSUM149 and rSUM190 cells compared to untreated parental cells. In addition, a significant decrease in XIAP levels (Fig. 8 and (1)) and cleavage were

observed i n t he p arental c ells unde rgoing apoptosis pos t-GW583340 t reatment. Immunofluorescent a nalysis of X IAP i n pa rental a nd r esistant S UM149 a nd S UM190 l ines confirm the immunoblot data. Analysis of the mean intensity of XIAP staining per pixel in the immunofluorescence da ta s howed t hat both resistant l ines ha d s ignificantly i ncreased X IAP expression c ompared t o t heir pa rental c ounterpart (Fig. 8; S UM149 vs . r SUM149, $p< 9x10^{8}$; SUM190 vs . r SUM190, p=0.0007). These data identify a mechanism of a poptotic d ysregulation, w hich predominantly includes increased XIAP, and not failure of GW583340 t o inhibit p -ErbB2 a nd p -AKT i n acquired resistance to the dual tyrosine kinase inhibitor in the IBC model studied.

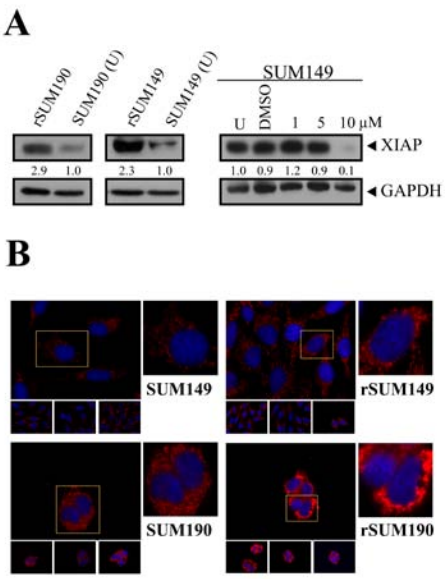

Figure 8. Western blot and immuofluorescence analysis of XIAP.

Potential Limitations

The tasks postulated in the grant proposal for year 1 have been completed. The following potential limitation was identified and an alternate strategy was developed.

The FLAG-tagged XIAP plasmid that was in the lab had adverse cytotoxic effects when transfected into the IBC cells in culture. Due to this reason, we obtained lentiviral expression plasmids from Dr. Colin Duckett (University of Michigan) to stably express XIAP so that transfection would no longer be a technical issue. To date, both SUM190 and SUM149 shXIAP, wtXIAP, and vector controls have been generated (Fig. 2-3).

Ongoing Studies

In addition to the studies outlined for year 2, we are currently generating mutant XIAP stable cell lines to further elucidate the mechanism of XIAP-mediated resistance to ErbB2 targeting agents. These mutants (single or double point mutations) interfere with the ability of XIAP to bind to caspases, Smac/Diablo and other proteins with an IAP binding motif (IBM), and to ubiquitinate other proteins (Fig. 1) (5). The stable expression of these XIAP mutants will be done in the SUM190 and SUM149 cell lines that have stable knockdown of XIAP expression using an shRNA XIAP plasmid (Fig. 2). These cells will then be treated with ErbB2 targeting agents and assessed for viability and apoptosis using Annexin V and 7-AAD staining in addition to caspase activity assays. These data will allow us to understand which domain(s) is important for the sensitivity and resistance of these cell lines to both trastuzumab and lapatinib.

Additional interesting studies that the laboratory would like to pursue within the scope of this grant proposal

Preliminary studies in our lab have shown that GW583340 causes an increase in reactive oxygen species (ROS) in cells that are sensitive to GW583340-induced apoptosis (Fig. 9). Previous studies in brain ischemia models and mouse embryonic fibroblasts (MEFs) have shown that XIAP plays a key role in the resistance of cells to oxidative stress-induced apoptosis (18-20). Therefore, our current hypothesis is that XIAP mediates resistance to GW583340 in part through decreasing oxidative stress-induced apoptosis. Objectives of this study include:

1. Demonstrating the novel mechanism of oxidative stress-induced apoptosis mediated by GW583340.
2. Determination of the mechanism of sensitivity and resistance to GW583340-mediated oxidative-stress induced apoptosis in parental and resistant SUM149 and SUM190 IBC cells.
3. Elucidating the domain(s) of XIAP that are potentially important for mediating the resistance of IBC cells to oxidative stress-induced apoptosis.

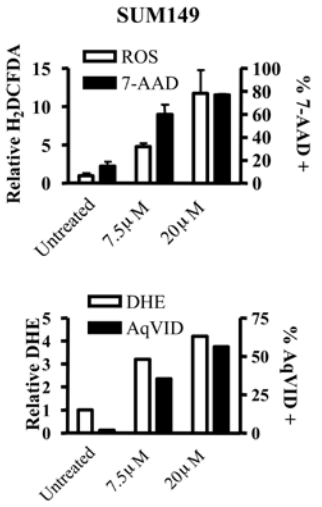

Figure 9. Expression of reactive oxygen species H2O2 and superoxides (using carboxy- H2DCFDA and dihydroethidium, respectively) post-treatment with GW583340 in SUM149 cells.

Key Research Accomplishments

1. We have successfully generated stably XIAP overexpressing SUM190 and SUM149 IBC lines in addition to the appropriate vector controls.
2. We have successfully generated GW583340-resistant SUM149 and SUM190 IBC cell lines that have similar doubling times to their parental counterparts and decreased apoptosis in the presence of GW583340.
3. We have successfully optimized a technique to visualize XIAP using immunofluorescent microscopy.

Reportable Outcomes

1. Poster P resentation a t t he 2009 C ell D eath P athways K eystone S ymposia, W histler, British Columbia

   Katherine M. Aird and Gayathri R. Devi. Stress-induced X-linked Inhibitor of Apoptosis Protein (XIAP) U pregulation in Lapatinib Resistant Inflammatory Breast C ancer C ell Lines

2. Manuscript in submission

   Katherine M. Aird, Rami Ghanayem, Sharon Peplinski, Herbert K. Lyerly, and Gayathri R. Devi. X-Linked Inhibitor of A poptosis P rotein Inhibits A poptosis i n Inflammatory Breast Cancer Cells w ith A cquired R esistance t o a n ErbB1/2 T yrosine K inase Inhibitor. (Molecular Cancer Therapeutics)

Conclusions

1. Inflammatory breast cancer is a highly aggressive disease that can often be targeted with ErbB2 targeting therapies such as trastuzumab and lapatinib.
2. De novo and acquired resistance to trastuzumab and lapatinib is common in women with IBC.
3. Decrease in XIAP expression correlates to sensitivity to both trastuzumab and GW583340 (a lapatinib analog).
4. Overexpression of XIAP correlates to de novo resistance to trastuzumab-mediated signaling changes.
5. Exogenous overexpression of XIAP reverses sensitivity of IBC cells to GW583340.
6. Cells with acquired resistance to GW583340 have decreased ErbB1 and ErbB2 signaling, however XIAP expression is increased.

Supported in part by DOD Predoctoral Grant W81XWH-08-1-0363

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Appendix I

Keystone Cell Death Pathways Abstract

Stress-induced X-linked Inhibitor of Apoptosis Protein (XIAP) Upregulation in Lapatinib Resistant Inflammatory Breast Cancer Cell Lines

Katherine M. Aird and Gayathri R. Devi, Departments of Pathology and Surgery, Comprehensive Cancer Ctr, Duke University Medical Center, Durham, NC, USA, 27710

Inflammatory breast cancer (IBC) is a highly aggressive form of locally advanced breast cancer that is often characterized by epidermal growth factor receptor (ErbB2/Her2) overexpression. Acquired resistance to clinically approved ErbB2-targeting agents (Trastuzumab and Lapatinib) is frequent. We have observed apoptotic dysregulation in IBC cells mediated by X-linked inhibitor of apoptosis protein (XIAP), one of the most potent anti-apoptotic proteins that is capable of inhibiting both mitochondrial and extrinsic pathways of apoptosis. XIAP overexpression distinctly correlates with acquired resistance to Trastuzumab and Lapatinib. The present study further identifies a potential mechanism of XIAP increase in IBC cellular models isolated from patient primary tumors. Analysis of XIAP mRNA expression by RT-PCR demonstrated no significant difference between the parental and Lapatinib-resistant IBC cells. Interestingly, XIAP has an internal ribosomal entry site (IRES) that can be used to translate XIAP during times of cellular stress when the canonical protein machinery is shut down. Transfection of an XIAP plasmid that is being specifically translated off the IRES showed a significant increase in XIAP protein expression in the Lapatinib-resistant lines versus their parental counterparts suggesting the role of XIAP in promoting cell survival even in the presence of stress/apoptosis-inducing agents like Lapatinib. Currently, adenoviral mediated overexpression constructs are being employed to characterize the interaction between the upregulated XIAP protein and other IAP family members. In summary, these data demonstrate a mechanism of XIAP up regulation in Lapatinib-resistant cells and may lead to more rational stress-related targets for use in combination with ErbB2 targeted agents.

Funded by American Cancer Society ACS-RSG-08-290-01-CCE and Department of Defense Predoctoral Grant W81XWH-08-1-0363

Appendix II

Manuscript in submission to *Molecular Cancer Therapeutics*

Katherine M. Aird[1 3], Rami B. Ghanayem[2], Sharon Peplinski[2], Herbert K. Lyerly[1 2 3], Gayathri R. Devi[1 2 3]

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Running Title: XIAP Confers Resistance to an ErbB1/2 Tyrosine Kinase Inhibitor

Keywords: IRES, embelin, survivin, FOXO3a, p-AKT

Abbreviations: BIR, baculoviral IAP repeat; ER, estrogen receptor; IAP, inhibitor of apoptosis protein; IBC, inflammatory breast cancer; IRES, internal ribosomal entry sequence; JNK, c-Jun N-terminal kinase; LABC, locally advanced breast cancer; MAPK, mitogen activated protein kinase; NF-κB, nuclear factor kappa B; UTR, untranslated region; XIAP, X-linked inhibitor of apoptosis protein

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Abstract

Inflammatory breast cancer (IBC) is a highly aggressive subtype of breast cancer that is often characterized by ErbB2 overexpression. ErbB2 targeting is clinically relevant using trastuzumab, an anti-ErbB2 antibody and lapatinib, a small molecule ErbB1/2 kinase inhibitor. However, acquired resistance is a common outcome even in those IBC patients who show an initial clinical response, which limits the efficacy of these agents. In the present study, using a clonal population of GW583340 (ErbB1/2 tyrosine kinase inhibitor)-resistant IBC cells, we have identified overexpression of an anti-apoptotic protein, XIAP, in acquired resistance to GW583340 in both ErbB2 overexpressing SUM190 and ErbB1 activated SUM149 cell lines derived from primary IBC tumors. A marked decrease in p-ErbB2, p-ErbB1, and downstream signaling was evident in the GW583340-resistant cells (rSUM190 and rSUM149) similar to the parental counterparts, suggesting the primary mechanism of action of GW583340 was not compromised in resistant cells. However, rSUM190 and rSUM149 cells growing in GW583340 had significant XIAP overexpression, sustained Bcl-2 and survivin levels, and resistance to GW583340-mediated apoptosis. The observed overexpression was identified to be an IRES-mediated translation of XIAP. XIAP downregulation in rSUM190 and rSUM149 cells using a small molecule inhibitor (embelin), which abrogates the interaction between XIAP and procaspase 9, resulted in decreased viability and increased apoptosis observed with annexin-V staining and nucleosome enrichment assay, demonstrating the dominance of XIAP expression in acquired resistance to GW583340. These studies establish the feasibility of development of an XIAP inhibitor that potentiates apoptosis for use in IBC patients with resistance to ErbB2-targeting.

Introduction

Apoptosis dysregulation is a fundamental characteristic of cancer that allows transformed cells to survive and proliferate (1, 2). In part, this failure is due to defects in caspase activity, the execution phase of apoptosis. The inhibitor of apoptosis proteins (IAP) are one of the major gene families that regulate caspase activation and programmed cell death (3). The family currently consists of eight members characterized by the presence of one or more baculoviral IAP repeat (BIR) domains and are highly conserved among mammalian and non-mammalian species (4).

In particular, one of the IAP proteins, X-linked inhibitor of apoptosis protein (XIAP), has been identified as the most potent caspase inhibitor to date (4). XIAP can bind and inhibit activation of procaspases 9, 7, and 3. This leads to inhibition of both intrinsic (mitochondrial) and extrinsic (death receptor-mediated) pathways of apoptosis (3), which is not evident with another prominent anti-apoptotic protein Bcl-2, which inhibits cytochrome c release from the mitochondria but does not directly bind to caspases (5). In addition, XIAP mRNA has an internal ribosomal entry sequence (IRES) (6), which has been identified to be upregulated during cellular stress (7-9). XIAP is expressed in almost all tissues and cell types (10) however, it is often overexpressed in tumors versus normal tissue (11), including breast cancer (12), and has been strongly linked to therapeutic resistance in cervical, ovarian, and prostate cancers (13, 14). In addition to its caspase-binding function, XIAP has been observed to regulate the activity of key survival factors like AKT, nuclear factor kappa B (NF-κB), and another IAP family member, survivin (15). As such, there is a growing interest in targeting XIAP, and inhibitors of XIAP are currently being developed for the clinic to help overcome resistance to mainstay therapies (16).

Recently, we reported a novel functional link between the epidermal growth factor receptor 2 (ErbB2) signaling pathway and XIAP in SUM190 cells, an ErbB2 overexpressing

inflammatory breast cancer (IBC) cell line resistant to trastuzumab, an ErbB2 targeting monoclonal antibody (17). IBC is an aggressive, fast-growing, and highly invasive cancer that is clinicopathologically distinct from a neglected locally advanced breast cancer (LABC) (18). IBC tumors are often resistant to chemo- and radio- therapy and therefore disease-free survival is poor (19, 20). ErbB2 is commonly overexpressed in IBC tumors (21) however, the development of acquired resistance to trastuzumab and lapatinib (a dual ErbB1/2 tyrosine kinase inhibitor) limits the clinical efficacy of these anti-ErbB2 therapeutic strategies (22-24). Clinical trials using lapatinib as a monotherapy have shown that it is effective in patients with ErbB2 overexpressing breast cancer that have been heavily pre-treated with other therapeutics including trastuzumab (25, 26), with response rates ranging from 7-35% (27). Interestingly, in IBC patients, lapatinib has a greater efficacy with response rates ranging from 50-100% (28, 29). However, clinical studies with lapatinib as a monotherapy also indicate that clinical responses are generally short-lived in breast cancer patients (30) and acquired resistance is common. Previously reported mechanisms of acquired resistance to lapatinib include activation of estrogen receptor (ER) signaling (24), upregulation of the antiapoptotic protein MCL-1 (31), and potentially the modulation of cancer cell metabolism (32). In the present study, we evaluated XIAP action in a model of acquired resistance to a lapatinib analog (GW583340) in both ErbB2 overexpressing and ErbB1 activated IBC cell lines wherein cells were chronically exposed GW583340, similar to patients receiving daily doses of lapatinib when given as a monotherapy. Continuous exposure to GW583340 for over 3 months converted the parental GW583340 sensitive IBC cells to being resistant to the apoptotic-inducing effects of the inhibitor. We identified XIAP overexpression to be the key difference between the parental GW583340 sensitive and both ErbB2 overexpressing and ErbB1 activated resistant IBC lines. This

overexpression was demonstrated to be a translational stress-related event mediated via translation of XIAP using its IRES in its 5' untranslated region (UTR). Further, XIAP downregulation using embelin (a small molecule inhibitor that interrupts the interaction between XIAP and procaspase 9) (33) caused reversal of GW583340 resistance in these IBC cells.

Materials and Methods

Cell culture

SUM149 and SUM190 cells were obtained from Asterand, Inc. (Detroit, MI). All cell lines were cultured as described previously (17). Laboratory grade lapatinib (herein called GW583340; Sigma) was dissolved in DMSO. GW583340-resistant SUM190 and SUM149 cells (rSUM190, rSUM149) were established by culturing cells in normal growth media supplemented with increasing concentrations of GW583340 (0.25-2.5 μM and 0.25-7.5 μM, respectively) for a minimum of 3 months. From then, both rSUM190 and rSUM149 cells were routinely cultured in 2.5 μM and 7.5 μM GW583340, respectively.

Western Immunoblot Analysis

Western immunoblot analysis was carried out as described previously (17). Membranes were incubated with primary antibodies against XIAP (BD Bioscience, San Jose, CA), procaspase 9 (NeoMarkers, Fremont, CA), actin, GAPDH, FOXO3a, Bcl-2 (Santa Cruz), survivin (R&D Systems, Minneapolis, MN), p-AKT (Ser473), AKT, p-ErbB2 (Tyr877), ErbB2, p-MAPK (Thr202/Tyr204), MAPK, ErbB1 (Cell Signaling), and total phosphotyrosine clone 4G10 (Upstate, Lake Placid, NY) overnight at 4 °C. Stripping of membranes for detection of total protein was done by stripping the same membrane as described previously (14). Densitometric analysis was performed using the NIH ImageJ software (http://rsb.info.nih.gov/ij/).

Immunofluorescent confocal microscopy

Cells were seeded onto cover slips (VWR, West Chester, PA) in dishes and allowed to reach 70% confluence. Cover slips were washed once with PBS, and fixed with 100% methanol at -20

°C for 20 min. After fixation, cells were blocked with 1% BSA/PBS at 37 °C for 30 min and incubated with XIAP antibody (BD Biosciences) for 1 h at room temperature. Cells were then washed three times with PBS for 5 min and incubated with R-PE-labeled secondary antibody (Southern Biotech, Birmingham, AL) for 1 h at room temperature. Cells were incubated for 1 min with 0.1 μg/ml Hoechst 33258 stain (Sigma). Finally, cells were washed three times with PBS, cover slips were inverted onto slides, sealed, and imaged on a Zeiss Axio Observer inverted widefield fluorescence microscope using a 63x/1.40 DIC Plan Apochromat objective. Images were captured on a Hamamatsu ORCA ER CCD camera (Hamamatsu Corporation, Bridgewater, NJ). The system was controlled by MetaMorph Software (Molecular Devices, Downingtown, PA). XIAP staining intensity was measured by using NIH ImageJ software.

Treatment of cells with agents for determination of viability and signaling

Cells were seeded in 6-well plates (Corning Incorporated, Corning, NY) and allowed to reach 70% confluence. Cells were treated for 24 h to 7 d in regular growth media with GW583340 (Sigma), 48 h with embelin (Sigma, dissolved in DMSO), or 24 h with LY294002 (Sigma, dissolved in DMSO). DMSO (at the same concentration as drug treatments) was used as a vehicle control. Cell viability was determined by trypan blue exclusion assay as described previously (17). Cells were harvested for western immunoblot analysis 24 h (GW583340, LY294002) or 48 h (embelin) after treatment.

Annexin-V staining for determination of apoptosis

Cells were seeded in their respective media in 6-well plates (Corning Incorporated) and allowed to reach 70% confluency. GW583340 (2.5 μM or 7.5 μM) and embelin (50 μM) were made in

regular growth media and cells were incubated for 48 h. DMSO (at the same concentration as drug treatments) was used as a vehicle control. Cells were then stained for annexin-V and PI using the Annexin-V Biotin Kit (Beckman Coulter, Fullerton, CA) as per manufacturer's instructions. At least twenty five thousand events were collected on a FACScalibur flow cytometer (Beckton Dickinson) and analyzed using Cellquest software (Beckton Dickinson).

Real time polymerase chain reaction

Quantitative real-time PCR was performed as described previously (17). β-actin was used as an internal control. ΔΔCT shows the difference between actin control and XIAP. Folds ($2^{-\Delta\Delta CT}$) represent changes normalized to the parental IBC cell. The primers designed to target XIAP and β-actin were described previously (17).

Construction of XIAP IRES luciferase plasmid and transfection of cells

The luciferase construct was constructed by inserting the 5' UTR of XIAP (kindly provided by Dr. Martin Holcik from the University of Ottawa) upstream of luciferase in the pGL3 Basic vector (Promega) and a CMV promoter upstream of the 5' UTR. For transfection of DNA, cells were seeded in their respective media in 24-well plates (Corning Incorporated) and allowed to reach 80-90% confluency. At that time, cells were transfected with 1.5 μg pGL3-hUTR.luc and 0.5 μg pRL-TK (Promega) DNA using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions. Cells were incubated for 24 h and lysed for luciferase activity assay.

Luciferase activity assay

Cells were lysed for 15 min in 500 µl luciferase lysis buffer (35 mg/ml Tris base, 0.69 5mg/ml CDTA, 10% glycerol, 0.5% Triton-X 100, pH 7.8) and 25 µl of the lysate was added to a 96-well plate (Corning). Luciferase activity was determined using a luminometer (Turner Biosystems, Sunnyvale, CA). Firefly or renilla luciferase substrate (1 mM luciferin or colelentrerazine [Gold Biotechnology, St. Louis, MO] in 15 mM $MgSO_4$, 15 mM K_2HPO_4, 4 mM EGTA, 1 mM DTT, 0.1 mM ATP) was added (100 µl) to wells and luciferase activity was read after 10 s.

Nucleosome Enrichment Assay

Cells were seeded in a 96-well plate (Corning Incorporated). Embelin (50 µM), staurosporine (5 µM, Sigma; dissolved in DMSO), and GW583340 were made in regular growth media. DMSO (at the same concentration as drug treatments) was used as a vehicle control. After 20 h incubation, nucleosome enrichment was determined by the Cell Death Detection ELISA^{PLUS} (Roche Applied Science; Mannheim, Germany) as per the manufacturer's instructions.

Statistical analysis

The statistical analyses were performed using Graphpad InStat Student's two tailed t-test and Anova (Turkey-Kramer multiple comparison test). Differences were considered significant at $p<0.05$.

Results

Development of a model of acquired resistance of IBC cells to a dual ErbB1/ErbB2 tyrosine kinase inhibitor

The effect of a laboratory grade lapatinib analog (herein called GW583340) was characterized in two well-established IBC cell lines isolated from primary IBC tumors (34): SUM190 [ErbB2 overexpressing, ER negative] and SUM149 [ErbB1 activated, ER negative]. Similar to previous reports using lapatinib (35, 36), both SUM190 and SUM149 were sensitive to the growth-inhibitory and apoptotic-inducing effects of GW583340 (Fig. 1). Since resistance to lapatinib monotherapy in patients treated with daily doses of lapatinib is commonly seen (30), GW583340-resistant lines (referred to here as rSUM190 and rSUM149) were established by chronic exposure of the parental SUM190 and SUM149 cells to increasing concentrations of GW583340 for greater than 3 months (see Materials and Methods). Data in Fig. 1 show that although rSUM190 and rSUM149 have similar doubling times to their parental counterparts, there is evidence of increased GW583340-induced apoptosis in the parental cells compared to the resistant isolates. SUM149 cells treated with 7.5 μM GW583340 were significantly growth inhibited ($p<0.005$) compared to rSUM149 cells growing in 7.5 μM GW583340 starting at 48 h and dramatically inhibited by 7 d (Fig. 1A). In apoptosis assays, SUM149 parental cells treated with 7.5 μM GW583340 had increased annexin-V positive cells compared to the resistant line growing in the same concentration (Fig. 1B). A similar trend was observed in the SUM190 cells, in which 2.5 μM GW583340 treatment decreased cell growth starting at 48 h post-treatment ($p<0.05$) and increased apoptosis compared to the rSUM190 counterpart (Fig. 1C, D).

Dysregulation in apoptotic pathway and not inhibition of ErbB signaling contributes to acquired resistance to GW583340

Evaluation of the effect of GW583340 on the ErbB signaling pathways in SUM190 and SUM149 IBC cells revealed that treatment with GW583340 caused a marked and comparable downregulation of p-ErbB1 in both parental SUM149 and resistant rSUM149 cells compared to untreated parental cells (Fig. 2A). Similarly, expression of p-ErbB2, p-AKT, and p-MAPK were inhibited in the GW583340-treated SUM190 and resistant rSUM190 cells, along with increase in total MAPK expression (Fig. 2B). These data suggest that the primary mechanism of GW583340 action as a dual ErbB1/2 kinase inhibitor (37) is not compromised in the resistant rSUM190 and rSUM149 cells.

Previous reports from our lab (17) and others (38) in IBC cells and patients have shown that sensitivity to lapatinib and GW583340 correlates with inhibition of XIAP and survivin, both of which are key members of the IAP family of proteins. Immunoblot analyses of GW583340 treated lysates for key anti-apoptotic proteins were conducted. Data in Fig. 3A reveal that survivin expression was not inhibited in the resistant IBC cells, consistent with previous studies in a non-IBC breast cell line (BT474) (24) and an ovarian carcinoma cell line (PEO1) (39), wherein high survivin expression was sustained in cells resistant to lapatinib.

Data in Fig. 3B also show that rSUM190 and rSUM149 cells had high Bcl-2 protein levels in comparison to the untreated parental cells. In contrast, a significant decrease in Bcl-2 expression was observed post-GW583340 treatment in the parental SUM190 and SUM149 cells undergoing apoptosis (Fig. 3C).

FOXO3a, a member of the forkhead family of transcription factors, has been shown to promote transcription of pro-apoptotic genes and inversely correlate with IAP expression (40). Further, FOXO3a seems to be involved in mediating resistance to lapatinib in an estrogen receptor (ER)-positive breast cancer model (24, 41). Data in Fig. 3D show that FOXO3a expression was indeed decreased in rSUM149 and rSUM190 cells resistant to GW583340-mediated apoptosis, although the difference was more marked in the rSUM190 cells.

Immunoblot analysis of XIAP protein levels (Fig. 4) in the IBC cells showed a 2-3 fold overexpression of XIAP in both rSUM149 and rSUM190 cells compared to untreated parental cells. In addition, a significant decrease in XIAP levels (Fig. 4A) (17) and cleavage (data not shown) were observed in the parental cells undergoing apoptosis post-GW583340 treatment. Immunofluorescent analysis of XIAP in parental and resistant SUM149 and SUM190 lines confirm the immunoblot data (Fig. 4B). Analysis of the mean intensity of XIAP staining per pixel in the immunofluorescence data showed that both resistant lines had significantly increased XIAP expression compared to their parental counterpart (Fig. 4C; SUM149 vs. rSUM149, $p<9x10^{8}$; SUM190 vs. rSUM190, $p= 0.0007$). These data identify a mechanism of apoptotic dysregulation, which predominantly includes increased XIAP, and not failure of GW583340 to inhibit p-ErbB2 and p-AKT in acquired resistance to the dual tyrosine kinase inhibitor in the IBC model studied.

XIAP overexpression in IBC cells with acquired resistance to GW583340 is due to IRES-mediated translation

To address the mechanism of XIAP upregulation in the GW583340-resistant IBC cells, we postulated that the increase must either be at the transcriptional level (i.e. more XIAP mRNA is being made in the resistant cells) or at the translational level wherein GW583340 treatment is potentially having an effect on stress-related pathways. XIAP has been identified to have an IRES element in its 5'UTR that has been previously shown to be a non-canonical translational start site in times of cellular stress (7-9). Real time RT-PCR analysis of XIAP mRNA showed that there was no significant change (SUM149 vs. rSUM149 $p=0.467$; SUM190 vs. rSUM190 $p=0.233$) in expression between GW583340-resistant cells and their parental counterparts (Fig. 5A). Characterization of the IRES-mediated translation of XIAP in the resistant and parental IBC cells was carried out by transiently transfecting a luciferase reporter wherein the 5' hUTR of XIAP was cloned immediately upstream of the firefly luciferase gene. Data in Fig. 5B reveal that both GW583340-resistant rSUM149 and rSUM190 cells had higher luciferase activity than their parental counterparts, when firefly luciferase expression was normalized to the co-transfected renilla luciferase plasmid (SUM149 vs. rSUM149, $p< 0.0005$; SUM190 vs. rSUM190, $p< 0.05$). These data demonstrate that the up regulation of XIAP in GW583340-resistant cells is likely due to the IRES-mediated translation of XIAP and not increase in XIAP mRNA.

Inhibition of XIAP function using a small molecule inhibitor causes apoptosis and overcomes GW583340 resistance

Since the data show that there is a significant increase in XIAP levels in IBC cells that have acquired resistance to GW583340-induced apoptotic response when chronically exposed to

GW583340, we evaluated the effect of inhibition of XIAP action by using a small molecule inhibitor, embelin. Embelin has been shown to prevent binding of XIAP to procaspase 9 and thereby increase caspase 9 activity and resultant apoptosis in cells (33). Since the primary mechanism of action of the dual ErbB1/2 inhibitor (i.e., inhibition of ErbB phosphorylation and PI3K/AKT signaling) is intact in the GW583340-resistant IBC cells, we compared embelin to a specific PI3K inhibitor (LY293002).

Representative immunoblots are shown in Fig. 6A to highlight the mechanism of action of embelin (decrease in procaspase 9) and LY294002 (decrease in p-AKT) in the IBC cells. Embelin treatment caused a marked decrease in viability in the GW583340-resistant cells (Fig. 6B), where increased XIAP expression was observed (Fig. 4). In contrast to embelin treatment, inhibition of ErbB2 signaling using a PI3K inhibitor or inhibition of survivin using siRNA (data not shown) had no effect on rSUM190 or rSUM149 cell viability, demonstrating the dominance of XIAP expression in acquired resistance to GW583340 (Fig. 6B). Apoptotic assays using annexin-V staining (Fig. 6C) revealed a marked increase in apoptotic cell populations in the rSUM149 (36.4%) and rSUM190 (40.8%) cells treated with embelin, comparable to the parental counterparts (34.8% and 33.5%, respectively). Nucleosome enrichment ELISA, which measures the amount of cytosolic nucleosomal fragmentation observed in cells undergoing apoptosis, (Fig. 6D) demonstrates that embelin causes increased nucleosome enrichment in the rSUM149 (4.6, $p<0.005$) and rSUM190 (7.3, $p<0.05$), comparable to the GW583340-sensitive parental SUM149 and SUM190 cells. In summary, these data demonstrate that inhibition of XIAP function causes apoptosis in the GW583340-resistant cell lines, similar to the apoptotic response seen in the parental cells, and overcomes the acquired resistance to chronic exposure to GW583340.

Discussion

We report herein apoptotic dysregulation correlating with XIAP overexpression in acquired resistance to GW583340 in both ErbB2 overexpressing SUM190 and ErbB1 activated SUM149 IBC cells lines derived from primary tumors of IBC patients. A marked decrease in p-ErbB2, p-ErbB1 and downstream signaling were evident in the GW583340-resistant cells (rSUM190 and rSUM149, respectively), similar to the parental counterparts suggesting that the primary mechanism of action of the dual ErbB1/2 tyrosine kinase inhibitor was not compromised in the resistant cells. However, rSUM190 and rSUM149 cells growing in GW583340 had significant XIAP overexpression and sustained Bcl-2 and survivin levels compared to the parental sensitive cells, wherein treatment with GW583340 caused significant apoptosis and decreased XIAP and Bcl-2. Overexpression of XIAP in GW583340 resistant cells was observed to be mediated by IRES-dependent translation. Inhibition of XIAP function using a small molecule inhibitor (embelin) that abrogates the inhibitory interaction between XIAP and procaspase 9 induced apoptosis in the GW583340-resistant IBC lines.

Lapatinib is a dual tyrosine kinase inhibitor and is therefore effective in tumors with either ErbB2 expression or ErbB1 expression. Both ErbB2 overexpressing and ErbB1 activated IBC cells were sensitive to the growth-inhibitory and apoptotic-inducing effects of GW583340 (a lapatinib analog). Evidence from the clinic has shown that IBC tumors are relatively more responsive to lapatinib than other breast cancer types (RR = 50% in IBC vs. <10% in non-IBC; (28, 29)), however the response to lapatinib is often short-lived and resistance is common (30). Two recent studies (24, 31) have shown that apoptotic signaling is an important mechanism of lapatinib resistance and the apoptotic pathways have been characterized to be dysregulated in IBC vs. other LABC types (42-45). Xia et al. (24) report that acquired resistance to lapatinib in

the ER-dependent non-IBC BT474 cells is due to increased activity of the transcription factor FOXO3a, which regulates ER downstream anti-apoptotic proteins such as survivin and Bcl-2. The other report demonstrated that MCL-1 (an anti-apoptotic member of the Bcl-2 family) was increased in colon cancer cells resistant to lapatinib (31). These studies support the idea that dysregulation of the apoptotic signaling pathway plays a key role in the resistance of cancer cells to lapatinib. In addition, a previous study in our lab has shown that XIAP expression correlates with resistance to trastuzumab in the ErbB2 overexpressing SUM190 IBC cells (17), further supporting the hypothesis that the anti-apoptotic signaling pathway is dysregulated in responses to ErbB2 targeting agents.

In the present study, a model of acquired resistance to a dual ErB1/2 tyrosine kinase inhibitor (lapatinib analog, GW583340) was generated since resistance to lapatinib monotherapy in patients treated with daily doses of lapatinib is commonly seen (30). The GW583340-resistant lines (rSUM190 and rSUM149) were established by chronic exposure of the parental SUM190 and SUM149 cells to the drug for greater than 3 months. It was shown that the primary mechanism of action of the tyrosine kinase inhibitor remained intact in the GW583340-resistant cellular model, and therefore we hypothesized that the apoptotic pathway was dysregulated. Similar to a previous report (24), Bcl-2 expression was increased and survivin expression was sustained in the resistant cell lines. In addition, XIAP was significantly upregulated in both ErbB2 overexpressing and ErbB1 activated lines resistant to GW583340. Both cell lines are ER negative, which may be why survivin and FOXO3a expression did not correlate as well to previous studies in ER positive breast cancer cell lines. Importantly, a majority of IBC tumors are ER negative (46), so this finding is clinically relevant. In addition, it has been reported that FOXO3a and XIAP expression show an inverse correlation (40). FOXO3a is upregulated by c-

jun N-terminal kinase (JNK) (40), which is negatively regulated by XIAP (47). It is therefore not surprising that FOXO3a expression was decreased in the resistant cells wherein XIAP was dramatically upregulated.

Since the data show that XIAP was specifically overexpressed in the resistant IBC lines, we tested the effects of the XIAP small molecule inhibitor embelin (33) in the IBC cellular model. The abrogation of the inhibitory interaction between XIAP and procaspase 9 by embelin was able to decrease cell viability and increase apoptosis in both the resistant ErbB2 overexpressing (SUM190) and ErbB1 activated (SUM149) IBC cells, demonstrating that inhibiting XIAP function is a potential target for breast cancers with acquired resistance to ErbB-targeting agents. In contrast to a previous report (24), inhibition of survivin by siRNA in these cells had no effect on viability (data not shown). This is consistent with the role of survivin as a non-traditional inhibitor of apoptosis as it has not been effectively shown to functionally inhibit caspases (48), although it has been sufficiently demonstrated to be a mitotic regulator (49), and may be why inhibition of this protein could not increase cell death in the IBC cell lines. Interestingly, XIAP has been previously shown to bind to and regulate the function of survivin (50), and therefore it is appealing to speculate the inhibition of both XIAP and survivin may be even more potent than inhibition of the molecules separately.

It is clear that apoptotic dysregulation is a critical factor in acquired lapatinib resistance in breast cancer. In addition, this study is the first to elucidate that XIAP overexpression corresponding with resistance to GW583340-induced apoptosis in the ErbB2 overexpressing and ErbB1 activated IBC cellular models is not due to increase in XIAP transcription but rather due to increased translation of XIAP via its IRES element present in its 5' UTR (7-9). These unique secondary structures can be used as a non-canonical translation start site during times of cellular

stress when traditional protein translation is shut down (6), identifying XIAP as a stress-related target for therapeutic intervention and establishing the feasibility of targeting XIAP in combination with lapatinib to enhance tumor apoptosis in IBC therapy.

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Figure Legends

Figure 1. Effect of GW583340 on proliferation and apoptosis in parental and resistant IBC cells. A, Effect of GW583340 on proliferation of parental and resistant SUM149 cells. Untreated cells were compared to cells treated with 7.5 μM GW583340 for indicated times. Cell count was assessed by trypan blue exclusion assay (n>2). ** p<0.005, rSUM149 vs. SUM149 treated with 7.5 μM GW583340. B, Annexin-V staining was assessed in untreated resistant and parental cells and compared to cells treated with 7.5 μM GW583340. Bars represent mean ± SEM (n=2), * p<0.05 C, Effect of GW583340 on proliferation of parental and resistant SUM190 cells. Untreated cells were compared to cells treated with 2.5 μM GW583340 for indicated times. Cell count was assessed by trypan blue exclusion assay (n>2).* p< 0.05, rSUM190 vs. SUM190 treated with 2.5 μM GW583340. D, Annexin-V staining was assessed in untreated resistant and parental cells and compared to cells treated with 2.5 μM GW583340. Bars represent mean ± SEM (n=2), * p<0.05

Figure 2. A, Immunoblot analysis of parental and resistant SUM149 cells with an antibody against p-ErbB1. The p-ErbB1 blot was stripped and reprobed for ErbB1 total protein. Numbers represent densitometric analysis of p-ErbB1 normalized to ErbB1. B, Immunoblot analysis of parental and resistant SUM190 cells with antibodies against p-ErbB2, p-MAPK, and p-AKT. GAPDH was used as a loading control for p-MAPK. Phospho blots were stripped and reprobed for corresponding total protein. Numbers represent densitometric analysis of p-ErbB2, p-MAPK, and p-AKT normalized to respective total protein and total MAPK normalized to GAPDH

Figure 3. Effect of acquired resistance to GW583340 on apoptotic signaling in IBC cells. A, Survivin immunoblot analysis of parental and resistant IBC cells. GADPH was used as a loading control. Numbers represent densitometric analysis of survivin normalized to GAPDH. B, Bcl-2 immunoblot analysis of parental and resistant IBC cells. GADPH was used as a loading control. Numbers represent densitometric analysis of Bcl-2 normalized to GAPDH. C, Bcl-2 immunoblot analysis of parental IBC cells treated with GW583340 for 24 h. GADPH was used as a loading control. Numbers represent densitometric analysis of Bcl-2 normalized to GAPDH. D, FOXO3a immunoblot analysis of parental and resistant IBC cells. GADPH was used as a loading control. Numbers represent densitometric analysis of FOXO3a normalized to GAPDH.

Figure 4. Effect of acquired resistance to GW583340 on XIAP in IBC cells. A, XIAP immunoblot analysis of parental and resistant SUM190 and SUM149 cells (left), and XIAP immunoblot analysis of parental SUM149 cells treated with GW583340 for 24 h (right). GADPH was used as a loading control. Numbers represent densitometric analysis of XIAP normalized to GAPDH. B, Representative fluorescent microscopy images of parental and resistant SUM149 and SUM190 cells probed with an XIAP antibody and counterstained with Hoechst. C, Mean XIAP staining intensity per pixel in parental and resistant IBC cells. Bars represent the average mean XIAP staining intensity per pixel ± SEM in over 20 single cells taken from 10 different fields.

Figure 5. Effect of acquired resistance to GW583340 on XIAP mRNA expression and protein translation in IBC cells. A, RT-PCR analysis of XIAP mRNA expression in parental and resistant IBC cells. β-actin was used as an internal control. p= ns (not significant) B, Luciferase

activity of parental and resistant SUM149 and SUM190 cells co-transfected with pGL3-hUTR(luc) and renilla plasmid (pRL-TK). Numbers represent the ratio of firefly luciferase activity to renilla luciferase activity and taken as a percentage of their respective untreated readout. * $p<0.05$, ** $p<0.0005$

Figure 6. Effect of inhibition of XIAP on viability, apoptosis and signaling in GW583340 resistant IBC cells. A, Immunoblot analysis of SUM190 cells treated with embelin (left panel) or LY294002 (40 μM; right panel) for 48 h with antibodies against procaspase 9 and p-AKT. GAPDH was used as a loading control for procaspase 9. The p-AKT blot was stripped and reprobed for AKT total protein. Numbers represent densitometric analysis of procaspase 9 normalized to GAPDH and p-AKT normalized to total protein. B, Effect of embelin and LY294002 on viability of parental and resistant SUM190 and SUM149 cells. Cells were treated for 48 h and assessed for viability via trypan blue exclusion assay. Bars represent mean ± SEM of duplicate values (n = 2). C, Annexin-V staining of parental and resistant SUM190 and SUM149 cells treated with Embelin (50 μM) for 48 h and stained with Annexin-V and PI to assess for apoptosis. Bars represent percentage of total population of cells that stained Annexin-V positive. Bars represent mean ± SEM of duplicate values (n = 2). D, Effect of embelin on nucleosome enrichment of parental and resistant SUM190 and SUM149 cells. Cells were treated with 5 μM staurosporine and 50 μM embelin for 20 h and assessed for nucleosome enrichment. Nucleosome enrichment was calculated by: (mU sample – Blank)/(mU untreated – Blank) *100. Bars represent mean ± SEM of duplicate values. *$p<0.05$, **$p<0.005$

Figure 1

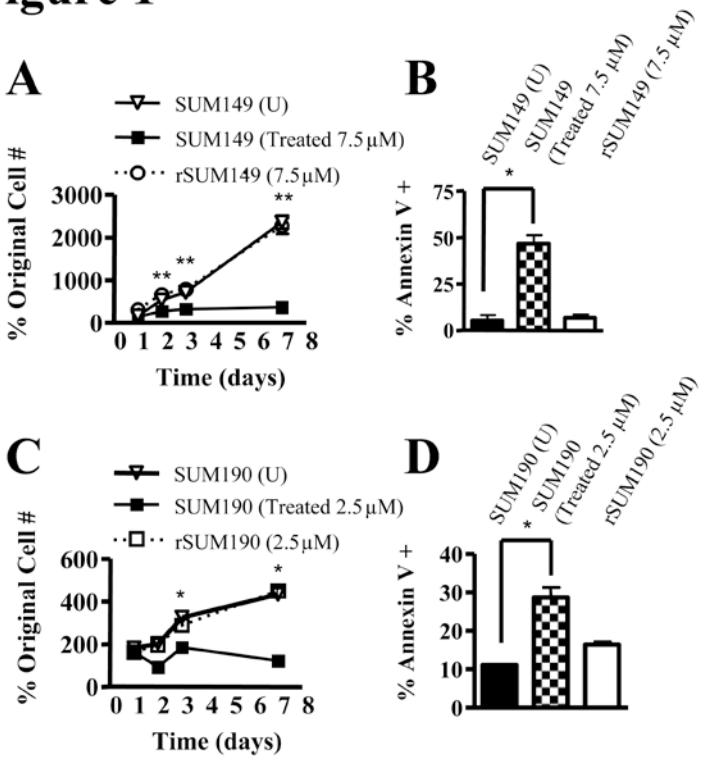

A
SUM149 (U)
SUM149 (Treated 7.5 µM)
rSUM149 (7.5 µM)
% Original Cell #
3000
2000
1000
0
0 1 2 3 4 5 6 7 8
Time (days)
**
**
**
B
SUM149 (U)
SUM149 (Treated 7.5 µM)
rSUM149 (7.5 µM)
*
% Annexin V +
75
50
25
0
C
SUM190 (U)
SUM190 (Treated 2.5 µM)
rSUM190 (2.5 µM)
% Original Cell #
600
400
200
0
0 1 2 3 4 5 6 7 8
Time (days)
*
*
D
SUM190 (U)
SUM190 (Treated 2.5 µM)
rSUM190 (2.5 µM)
*
% Annexin V +
40
30
20
10
0

Figure 2

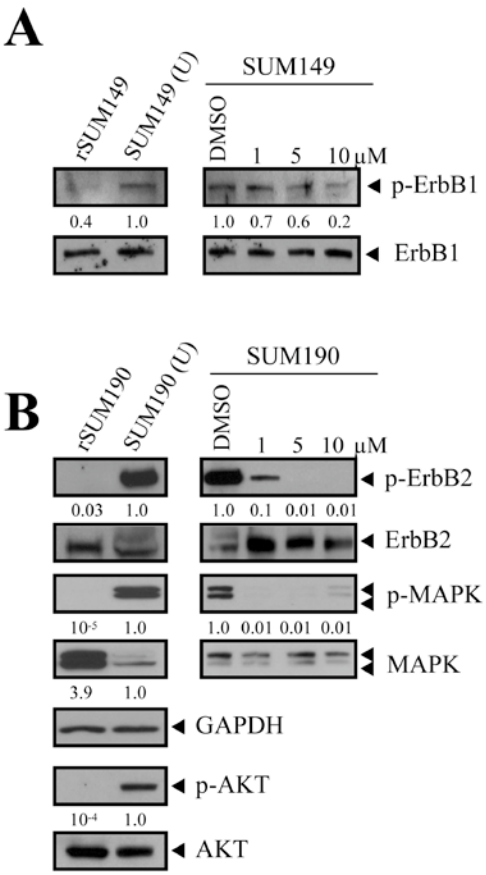

A
rSUM149
SUM149 (U)
SUM149
DMSO
1
5
10 μM
p-ErbB1
0.4
1.0
1.0
0.7
0.6
0.2
ErbB1
B
rSUM190
SUM190 (U)
SUM190
DMSO
1
5
10 μM
p-ErbB2
0.03
1.0
1.0
0.1
0.01
0.01
ErbB2
p-MAPK
10-5
1.0
1.0
0.01
0.01
0.01
MAPK
3.9
1.0
GAPDH
p-AKT
10-4
1.0
AKT

Figure 3

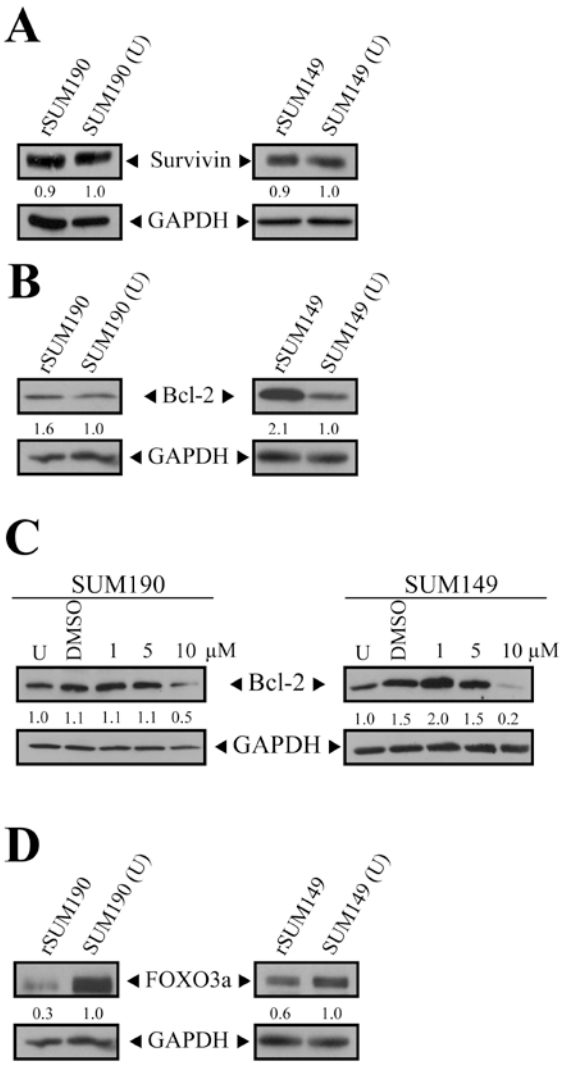

A
rSUM190
SUM190 (U)
rSUM149
SUM149 (U)
Survivin
0.9 1.0 0.9 1.0
GAPDH
B
rSUM190
SUM190 (U)
rSUM149
SUM149 (U)
Bcl-2
1.6 1.0 2.1 1.0
GAPDH
C
SUM190
SUM149
U DMSO 1 5 10 μM
U DMSO 1 5 10 μM
Bcl-2
1.0 1.1 1.1 1.1 0.5
1.0 1.5 2.0 1.5 0.2
GAPDH
D
rSUM190
SUM190 (U)
rSUM149
SUM149 (U)
FOXO3a
0.3 1.0 0.6 1.0
GAPDH

Figure 4

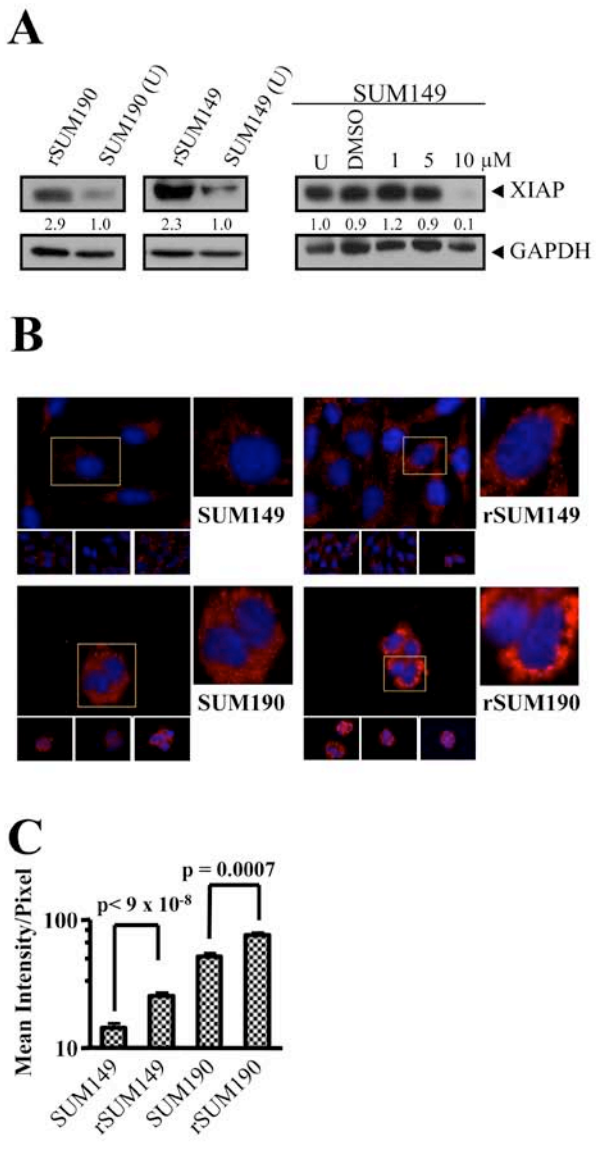

A
rSUM190
SUM190 (U)
rSUM149
SUM149 (U)
SUM149
U
DMSO
1
5
10 μM
XIAP
GAPDH
2.9
1.0
2.3
1.0
1.0
0.9
1.2
0.9
0.1
B
SUM149
rSUM149
SUM190
rSUM190
C
Mean Intensity/Pixel
p< 9 x 10-8
p = 0.0007
100
10
SUM149
rSUM149
SUM190
rSUM190

Figure 5

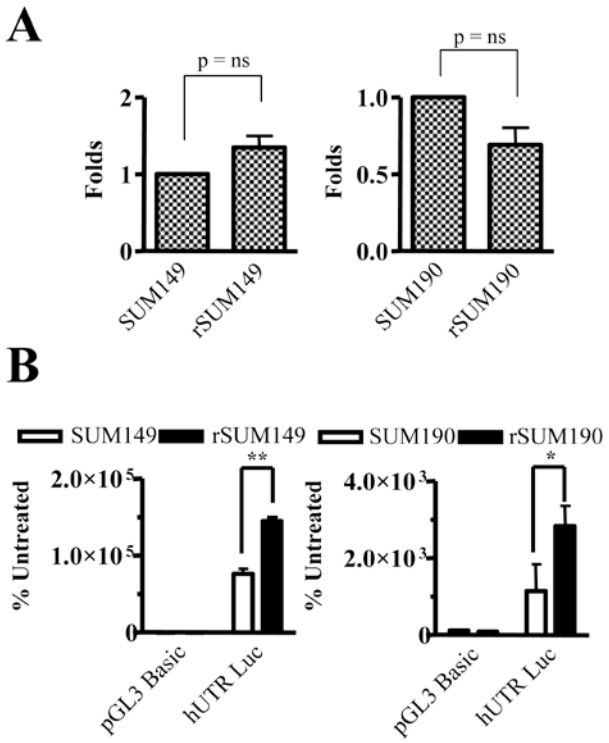
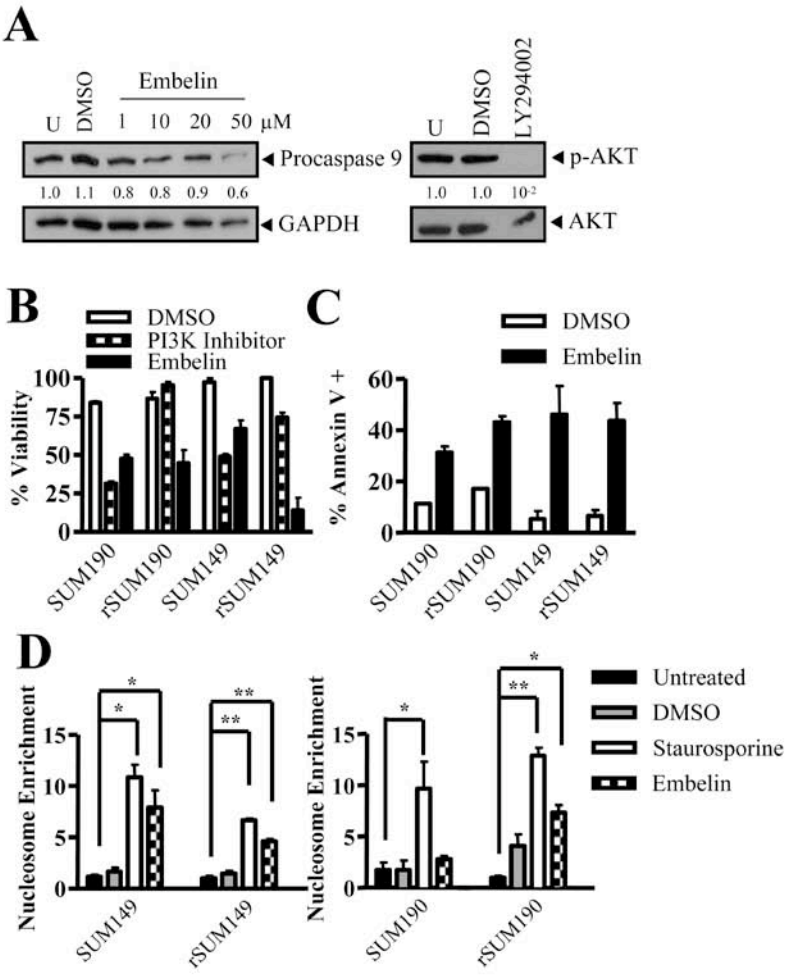

A
p = ns
Folds
2
1
0
SUM149
rSUM149
p = ns
Folds
1.0
0.5
0.0
SUM190
rSUM190
B
SUM149
rSUM149
SUM190
rSUM190
**
*
% Untreated
2.0×10 5
1.0×10 5
0
% Untreated
4.0×10 3
2.0×10 3
0
pGL3 Basic
hUTR Luc
pGL3 Basic
hUTR Luc

Figure 6


A
U
DMSO
Embelin
1
10
20
50 μM
Procaspase 9
1.0 1.1 0.8 0.8 0.9 0.6
GAPDH
U
DMSO
LY294002
p-AKT
1.0 1.0 10-2
AKT
B
DMSO
PI3K Inhibitor
Embelin
% Viability
100
75
50
25
0
SUM190
rSUM190
SUM149
rSUM149
C
DMSO
Embelin
% Annexin V +
60
40
20
0
SUM190
rSUM190
SUM149
rSUM149
D
Nucleosome Enrichment
15
10
5
0
SUM149
rSUM149
Nucleosome Enrichment
SUM190
rSUM190
Untreated
DMSO
Staurosporine
Embelin